

***Lasiodiplodia cinnamomi* sp. nov. from *Cinnamomum camphora* in China**

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ABSTRACT—A new species, *Lasiodiplodia cinnamomi*, is described as a pathogen causing branch canker of *Cinnamomum camphora* in Southern China. Morphology and phylogenetic analyses of ITS, RPB2, TEF1- α , and TUB2 sequence data support the position of the new species in *Lasiodiplodia*.

KEY WORDS —*Botryosphaeriaceae*, phylogeny, taxonomy

Introduction

Cinnamomum camphora is one of the principle tree species of China for ornamentation and timber (Li & al. 2007). In China, *Cinnamomum camphora* has been cultivated widely as street trees for its fast growth, fine wood, beautiful form, disease and insect resistance, resistance to pollution and acid rain (Wei 2008). During our investigation of plant diseases in Jiangsu Province, we found that several *Cinnamomum camphora* trees were infected by botryosphaeriaceous fungi, leading to tree death.

Fungi in *Botryosphaeriaceae* Theiss. & Syd. inhabit a wide diversity of hosts and substrates, including most economically and ecologically important trees and crops. *Lasiodiplodia* can be distinguished from related genera easily by the presence of pycnidial paraphyses and longitudinal striations on mature conidia (Phillips & al. 2013). In addition, the use of a combination of ITS and

TEF1- α sequences in the phylogenetic analysis of *Diplodia*, *Lasiodiplodia* and *Neofusicoccum* was suggested to separate species, because morphology is not a reliable character for species differentiation (Phillips & al. 2013).

Materials & methods

Samples and isolates

Fresh samples of *Lasiodiplodia* were collected from infected branches on *Cinnamomum camphora* in Jiangsu Province, China. Following a modified method of Fan & al. (2014), single conidia were isolated onto the surface of 1.8% PDA and incubated at 25 °C for up to 24 h. Single germinating conidia were removed onto fresh PDA plates. Two strains were used in this phylogenetic analysis. Specimens and isolates of the new species were deposited in the Museum of Beijing Forestry University, Beijing, China (BJFC). Axenic cultures are maintained in the China Forestry Culture Collection Center, Beijing, China (CFCC).

Morphological studies

Morphological observation was based on features of fruiting bodies produced on diseased plant tissues and cultural characteristics. Fruiting bodies were observed following the method of Slippers & al. (2013) and 50 spores were selected randomly for measurement using a Leica DM 2500 compound light microscope. Morphological or cultural characteristics, including colony color and texture, were observed on PDA after incubation at 25 °C, and recorded at 3 and 7 days.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from 7-day old colonies grown on PDA using a modified CTAB method (Doyle & Doyle 1990). DNA fragment size was checked with electrophoresis on 1% agarose gels, and the quality was measured by Thermo NanoDrop™ 2000 according to the user's manual (Desjardins & al. 2009). For the sequence data amplification of ITS, TEF1- α , TUB2, and RPB2, DNA Engine (PTC-200) Peltier Thermal Cycler was used with primers ITS5/ITS4 (White & al. 1990), EF1-688F/EF1-986R (Alves & al. 2008, Carbone & Kohn 1999), Bt2a/Bt2b (Glass & Donaldson 1995), and RPB2-LasF/RPB2-LasR (Cruywagen & al. 2017). The PCR amplification products were estimated visually by electrophoresis in 1.5% agarose gels. DNA sequencing was performed using an ABI PRISM® 3730XL DNA Analyzer with a BigDye® Terminator Kit v.3.1 at the Shanghai Invitrogen Biological Technology Company Limited (Beijing, China).

DNA sequence analysis

Sequences from this study and reference sequences obtained from GenBank (TABLE 1) were aligned using MAFFT v.6 (Katoh & Toh 2010) and edited manually using MEGA6 (Tamura & al. 2013). Phylogenetic analyses were generated by PAUP v.4.0b10 for maximum parsimony (MP) analysis (Swofford 2003), PhyML v.7.2.8 for maximum likelihood (ML) analysis (Guindon & al. 2010), and MrBayes v.3.1.2 for

TABLE 1. Sequences of *Lasiodiplodia* spp. and *Diplodia mutila* outgroup used in phylogenetic analyses

SPECIES	STRAIN	HOST	ORIGIN	GENBANK NUMBER			
				ITS	EF1- α	TUB	RPB2
<i>Diplodia mutila</i>	CMW 7060	<i>Fraxinus</i>	Netherlands	AY236955	AY236904	AY236933	EU339574
<i>L. avicenniae</i>	CMW41467*	<i>Avicennia</i>	South Africa	KP860835	KP860680	KP860758	KU587878
	LAS199	<i>Avicennia</i>	South Africa	KU587957	KU587947	KU587868	KU587880
<i>L. brasiliensis</i>	CMM 4015*	<i>Mangifera</i>	Brazil	JX464063	JX464049	—	—
	CMM 4469	<i>Anacardium</i>	Brazil	KT325574	KT325580	—	—
<i>L. bruguiera</i>	CMW41470*	<i>Bruguiera</i>	South Africa	KP860833	KP860678	KP860756	KU587875
	CMW 42480	<i>Bruguiera</i>	South Africa	KP860832	KP860677	KP860755	KU587876
<i>L. caatinguensis</i>	CMM 1325*	<i>Citrus</i>	Brazil	KT154760	KT008006	KT154767	—
	IBL 381	<i>Spondias</i>	Brazil	KT154757	KT154751	KT154764	—
<i>L. chinensis</i>	CGMCC 3.18061*	Unknown	China	KX499889	KX499927	KX500002	KX499965
	CGMCC 3.18044	<i>Vaccinium</i>	China	KX499875	KX499913	KX499988	KX499951
<i>L. cinnamomi</i>	CFCC 51997*	<i>Cinnamomum</i>	China	MG866028	MH236799	MH236797	MH236801
	CFCC 51998	<i>Cinnamomum</i>	China	MG866029	MH236800	MH236798	MH236802
<i>L. citricola</i>	CBS 124707*	<i>Citrus</i>	Iran	GU945354	GU945340	KU887505	KU696351
	CBS 124706	<i>Citrus</i>	Iran	GU945353	GU945339	KU887504	KU696350
<i>L. crassispora</i>	CMW 13448	<i>Eucalyptus</i>	Venezuela	DQ103552	DQ103559	KU887504	KU696350
	CBS 118741*	<i>Santalum</i>	Australia	DQ103550	DQ103557	KU887506	KU696353
<i>L. euphorbiaceicola</i>	CMW 33350	<i>Adansonia</i>	Botswana	KU887149	KU887026	KU887455	KU696346
	CMW 36231	<i>Adansonia</i>	Zimbabwe	KU887187	KU887063	KU887494	KU696347
<i>L. exigua</i>	CBS 137785*	<i>Retama</i>	Tunisia	KJ638317	KJ638336	KU887509	KU696355
	BL 184	<i>Retama</i>	Tunisia	KJ638318	KJ638337	—	—
<i>L. gilanensis</i>	CBS 124704*	—	Iran	GU945351	GU945342	KU887511	KU696357
	CBS 124705	—	Iran	GU945352	GU945341	KU887510	KU696356

<i>L. gonubiensis</i>	CMW 14077*	<i>Syzygium</i>	South Africa	AY639595	DQ103566	DQ458860	KU696359
	CMW 14078	<i>Syzygium</i>	South Africa	AY639594	DQ103567	EU673126	KU696358
<i>L. gravistriata</i>	CMM 4564*	<i>Anacardium</i>	Brazil	KT250949	KT250950	—	—
	CMM 4565	<i>Anacardium</i>	Brazil	KT250947	KT266812	—	—
<i>L. hormozganensis</i>	CBS 124709*	<i>Olea</i>	Iran	GU945355	GU945343	KU887515	KU696361
	CBS 124708	<i>Mangifera</i>	Iran	GU945356	GU945344	KU887514	KU696360
<i>L. hyalina</i>	CGMCC 3.17975	<i>Acacia</i>	China	KX499879	KX499917	KX499992	KX499955
	CGMCC 3.18383	Tree	China	KY767661	KY751302	KY751299	KY751296
<i>L. iraniensis</i>	IRAN 1520C	<i>Salvadora</i>	Iran	GU945348	GU945336	KU887516	KU696363
	IRAN 1502C	<i>Juglans</i>	Iran	GU945347	GU945335	KU887517	KU696362
<i>L. laeliocattleyae</i>	BOT-10*	<i>Mangifera</i>	Egypt	JN814397	JN814424	KU887508	KU696354
	BOT-29	<i>Mangifera</i>	Egypt	JN814401	JN814428	—	—
<i>L. lignicola</i>	CBS 134112*	Wood	Thailand	JX646797	KU887003	JX646845	KU696364
	MFLUCC 11-0656	Wood	Thailand	JX646798	—	JX646846	—
<i>L. macrospora</i>	CMM 3833*	<i>Jatropha</i>	Brazil	KF234557	KF226718	KF254941	—
<i>L. mahajangana</i>	CMW 27801*	<i>Terminalia</i>	Madagascar	FJ900595	FJ900641	FJ900630	KU696365
	CMW 27818	<i>Terminalia</i>	Madagascar	FJ900596	FJ900642	FJ900631	KU696366
<i>L. margaritacea</i>	CBS 122519*	<i>Adansonia</i>	Australia	EU144050	EU144065	KU887520	KU696367
	CBS 122065	<i>Adansonia</i>	Australia	EU144051	EU144066	—	—
<i>L. mediterranea</i>	CBS 137783*	<i>Quercus</i>	Italy	KJ638312	KJ638331	KU887521	KU696368
	CBS 137784	<i>Vitis</i>	Italy	KJ638311	KJ638330	KU887522	KU696369
<i>L. missouriana</i>	CBS 128311*	<i>Vitis</i>	USA	HQ288225	HQ288267	HQ288304	KU696370
	CBS 128312	<i>Vitis</i>	USA	HQ288226	HQ288268	HQ288305	KU696371
<i>L. parva</i>	CBS 456.78*	<i>Manihot</i> soil	Colombia	EF622083	EF622063	KU887523	KU696372
	CBS 495.78	<i>Manihot</i> soil	Colombia	EF622084	EF622064	EU673114	KU696373

<i>L. plurivora</i>	STE-U 5803*	<i>Prunus</i>	South Africa	EF445362	EF445395	KU887524	KU696374
	STE-U 4583	<i>Vitis</i>	South Africa	AY343482	EF445396	KU887525	KU696375
<i>L. pontae</i>	CMM1277	<i>Spondias</i>	Brazil	KT151794	KT151791	KT151797	—
<i>L. pseudotheobromae</i>	CBS 116459*	<i>Gmelina</i>	Costa Rica	EF622077	EF622057	EU673111	KU696376
	CGMCC 3.18047	<i>Pteridium</i>	China	KX499876	KX499914	KX499989	KX499952
<i>L. pyriformis</i>	CBS 121770*	<i>Acacia</i>	Namibia	EU101307	EU101352	KU887527	KU696378
	CBS 121771	<i>Acacia</i>	Namibia	EU101308	EU101353	KU887528	KU696379
<i>L. rubropurpurea</i>	WAC 12535*	<i>Eucalyptus</i>	Australia	DQ103553	DQ103571	EU673136	KU696380
	WAC 12536	<i>Eucalyptus</i>	Australia	DQ103554	DQ103572	KU887530	KU696381
<i>L. sterculiae</i>	CBS 342.78*	<i>Sterculia</i>	Germany	KX464140	KX464634	KX464908	KX463989
<i>L. subglobosa</i>	CMM 3872*	<i>Jatropha</i>	Brazil	KF234558	KF226721	KF254942	—
	CMM 4046	<i>Jatropha</i>	Brazil	KF234560	KF226723	KF254944	—
<i>L. thailandica</i>	CGMCC 3.18382	<i>Podocarpus</i>	China	KY767662	KY751303	KY751300	KY751297
	CGMCC 3.18384	<i>Albizia</i>	China	KY767663	KY751304	KY751301	KY751298
<i>L. theobromae</i>	CBS 164.96*	Fruit	New Guinea	AY640225	AY640258	KU887532	KU696383
	CBS 111530	—	—	EF622074	EF622054	KU887531	KU696382
<i>L. venezuelensis</i>	WAC 12539*	<i>Acacia</i>	Venezuela	DQ103547	DQ103568	KU887533	KU696384
	WAC 12540	<i>Acacia</i>	Venezuela	DQ103548	DQ103569	KU887534	—
<i>L. viticola</i>	CBS 128313*	<i>Vitis</i>	USA	HQ288227	HQ288269	HQ288306	KU696385
	UCD 2604MO	<i>Vitis</i>	USA	HQ288228	HQ288270	HQ288307	KU696386
<i>L. vitis</i>	CBS 124060*	<i>Vitis</i>	Italy	KX464148	KX464642	KX464917	KX463994

New sequences are set in bold font. Ex-type/ex-epitype isolates are marked by *.

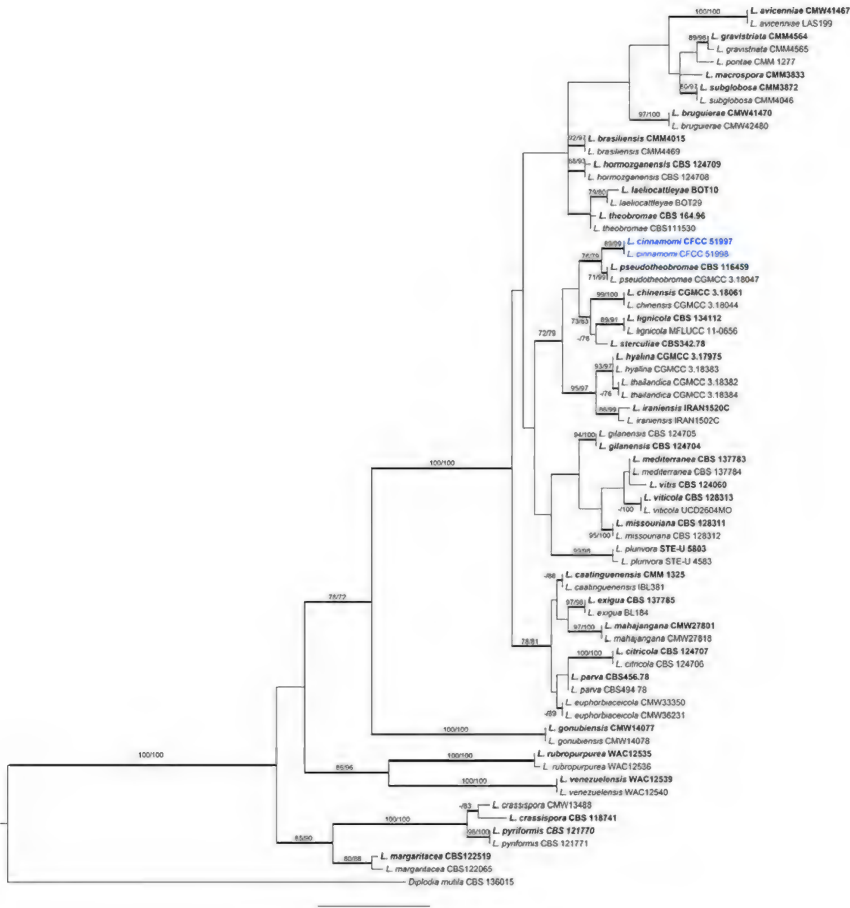


PLATE 1. Phylogram of ITS, RPB2, TEF1- α , and TUB2 regions based on MP analysis. (The ML and BI analyses produced congruent trees.) Values above the branches indicate maximum parsimony bootstrap (MP BP $\geq 70\%$) and maximum likelihood bootstrap (ML BP $\geq 70\%$). Thickened branches represent posterior probabilities ≥ 0.90 from BI. Scale bar = 20 nucleotide substitutions. Newly generated sequences are set in blue font. Ex-type strains are in bold font.

Bayesian Inference (BI) (Ronquist & Huelsenbeck 2003). The phylogenetic tree was constructed using the combined multi-gene dataset to compare our new *Lasiodiplodia* sequences with other ex-type material sequenced in recent studies (Alves & al. 2007, Phillips & al. 2013, Slippers & al. 2013, Linaldeddu & al. 2015).

We selected *Diplodia mutila* (Fr.) Mont. (CBS 136015) as outgroup (Phillips & al. 2013). Trees are shown using FigTree v.1.3.1 (Rambaut & Drummond 2010). MP analysis was performed by a heuristic search option of 1000 random-addition sequences with a tree bisection and reconnection (TBR) algorithm. Maxtrees were set to 5000, branches of zero length were collapsed and all equally parsimonious trees were saved. Other calculated parsimony scores were tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency (RC). ML analysis was also performed with a GTR site substitution model (Guindon & al. 2010). The branch support was evaluated with a bootstrapping (BS) method of 1000 replicates (Hillis & Bull 1993). For the BI analysis, a Markov Chain Monte Carlo (MCMC) algorithm was performed (Rannala & Yang 1996). The models of evolution were estimated by MrModeltest v.2.3 (Posada & Crandall 1998). Sequence data were deposited in GenBank (TABLE 1). The multilocus sequences alignment file was deposited in TreeBASE (www.treebase.org) as accession S21480. The taxonomic novelty was deposited in MycoBank (Crous & al. 2004).

Results

Molecular phylogeny

The combined gene (ITS, TEF1- α , TUB2, and RPB2) data matrix contains 68 strains from 36 *Lasiodiplodia* species, and one outgroup strain. The statistics for the parsimony analysis revealed that from the remaining 1611 characters, 1300 characters were constant, 82 variable characters were parsimony-uninformative and 229 characters were parsimony informative. The MP analysis yielded one most parsimonious tree (TL = 529, CI = 0.688, RI = 0.858, RC = 0.591), shown in PLATE 1. The phylogenetic tree obtained from ML and Bayesian analyses with the MCMC algorithm was consistent with the illustrated MP tree. Separate bootstrap values >50% for the MP and the ML analyses are presented above the nodes (as “MP/ML”); and BI values ≥ 0.90 are indicated by thickened branches.

Taxonomy

Lasiodiplodia cinnamomi C.M. Tian & Ning Jiang, sp. nov.

PLATE 2

MYCOBANK MB 822574

Differs from *Lasiodiplodia pseudotheobromae* by its smaller conidia.

TYPE: China, Jiangsu Prov., Gaoyou City, 32°47'25"N 119°28'12"E, 1 m asl, on twigs and branches of *Cinnamomum camphora* (L.) J. Presl (*Lauraceae*), 12 February 2017, coll. N. Jiang (Holotype, BJFC-S1374; ex-type culture CFCC 51997; GenBank ITS MG866028, EF1- α MH236799, TUB MH236797, RPB2 MH236801).

ETYMOLOGY: *cinnamomi* (Lat.), named after the host genus.

Conidiomata formed on hosts, pycnidial, multilocular, dark brown to black, semi-immersed in the host becoming erumpent when mature. Paraphyses



PLATE 2. Morphology of *Lasiodiplodia cinnamomi* from *Cinnamomum camphora* (BJFC-S1374). A: Habit of conidiomata on stem; B: Transverse sections through conidiomata; C: Longitudinal sections through conidiomata; D: Colonies on PDA at 3 days; E: Colonies on PDA at 10 days; F: Transverse sections through conidiomata; G–I: Conidiogenous layer with conidia developing on conidiogenous cells between paraphyses; J: Conidiogenous cells; K, L: Immature conidia; M, N: Mature conidia in two different focal planes to show the longitudinal striations. Scale bars: B, C, F = 100 μ m, G–N = 10 μ m.

hyaline, cylindrical, aseptate, sometimes branched, ends rounded, $\leq 106 \times 3\text{--}4$ μ m, arising amongst the conidiogenous cells. Conidiogenous cells hyaline, smooth, cylindrical, slightly swollen at the base, holoblastic, proliferating percurrently to form one or two closely spaced annellations, $10.4\text{--}13.6 \times 2.4\text{--}6.3$ μ m. Conidia ellipsoidal, apex and base rounded, widest at the middle, thick-walled, initially hyaline and aseptate and remaining so for a long time,

becoming 1-septate and dark brown only some time after release from the conidiomata, with melanin deposits on the inner surface of the wall arranged longitudinally giving a striate appearance to the conidia, (17.5–)18.7–21.1 (–22.4) $\times$ (11.5–)12.7–14.1 (–15.5) μm , 95% confidence limits = 18.7–21.1 $\times$ 12.7–14.1 μm ($\bar{x} \pm \text{S.D.} = 19.9 \pm 1.2 \times 13.4 \pm 0.7 \mu\text{m}$, l/w ratio = 1.5 ± 0.1). Colonies initially white to light-brown with fluffy, aerial mycelium, becoming grey on the surface after 3 days; reverse side of the colonies dark-brown. Sexual morph not observed.

HOST/DISTRIBUTION: on branches of *Cinnamomum camphora* in China.

ADDITIONAL SPECIMEN EXAMINED: CHINA, JIANGSU PROV., Gaoyou City, 32°47'25"N 119°28'12"E, 1 m asl, on twigs and branches of *Cinnamomum camphora*, 12 February 2017, coll. N. Jiang (BJFC-S1375; ex-type culture CFCC 51998; GenBank ITS MG866029, EF1- α MH236800, TUB MH236798, RPB2 MH236802).

NOTE: Two strains representing *L. cinnamomi* clustered in a supported clade and this species is considered to be a distinct species based on molecular data. The most closely related species in the phylogram is *L. pseudotheobromae* A.J.L. Phillips & al., which differs morphologically by its larger conidia (22.5–33 $\times$ 13.5–20 μm ; Alves & al. 2008).

Discussion

In this study, two strains of *Lasiodiplodia* associated with camphor canker disease in China were identified. Species identification was supported by morphology and multigene DNA data (ITS, TEF1- α , TUB, and RPB2), which indicated that *Lasiodiplodia cinnamomi* represents a distinct species (PLATE 1). Although *L. cinnamomi* represents a sister group to *L. pseudotheobromae*, the two species can be distinguished by conidial size. Hence, we regard this taxon as a novel species of *Lasiodiplodia*. It should be noted that characteristic mature conidia of *L. cinnamomi* are not easily observed from conidiomata on bark, because the conidia have already been released when mature.

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